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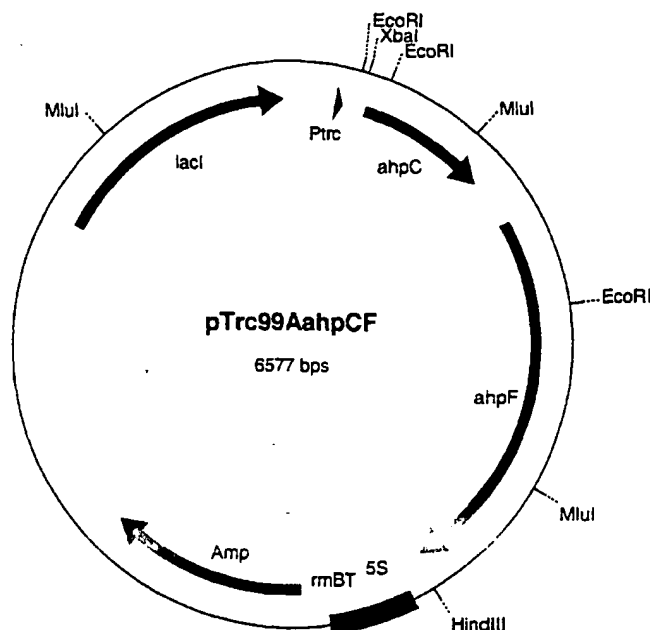
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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.



**Process for the Preparation of L-Amino Acids using Strains
of the Enterobacteriaceae Family**

Field of the Invention

This invention relates to a process for the preparation of
5 L-amino acids, in particular L-threonine, using strains of
the Enterobacteriaceae family in which at least one or more
of the genes chosen from the group consisting of *ahpC* and
ahpF is (are) enhanced.

Prior Art

10 L-Amino acids, in particular L-threonine, are used in human
medicine and in the pharmaceuticals industry, in the
foodstuffs industry and very particularly in animal
nutrition.

It is known to prepare L-amino acids by fermentation of
15 strains of Enterobacteriaceae, in particular *Escherichia*
coli (*E. coli*) and *Serratia marcescens*. Because of their
great importance, work is constantly being undertaken to
improve the preparation processes. Improvements to the
process can relate to fermentation measures, such as e.g.
20 stirring and supply of oxygen, or the composition of the
nutrient media, such as e.g. the sugar concentration during
the fermentation, or the working up to the product form, by
e.g. ion exchange chromatography, or the intrinsic output
properties of the microorganism itself.

25 Methods of mutagenesis, selection and mutant selection are
used to improve the output properties of these
microorganisms. Strains which are resistant to
antimetabolites, such as e.g. the threonine analogue α -
amino- β -hydroxyvaleric acid (AHV), or are auxotrophic for
30 metabolites of regulatory importance and produce L-amino
acid, such as e.g. L-threonine, are obtained in this
manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

Summary of the Invention

The invention provides a process for the preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which at least one or more of the nucleotide sequence(s) which code(s) for the *ahpC* and *ahpF* genes is (are) enhanced.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or

protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is
5 in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

10 The process is characterized in that the following steps are carried out:

- 15 a) fermentation of microorganisms of the Enterobacteriaceae family in which one or more of the genes chosen from the group consisting of *ahpC* and *ahpF* is (are) enhanced,
- b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- 20 c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose,
25 fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera *Escherichia*, *Erwinia*, *Providencia* and *Serratia*. The genera *Escherichia* and *Serratia* are
30 preferred. Of the genus *Escherichia* the species *Escherichia coli* and of the genus *Serratia* the species *Serratia marcescens* are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*,
15 are, for example

- Serratia marcescens HNr21
- Serratia marcescens TLR156
- Serratia marcescens T2000.

Strains from the Enterobacteriaceae family which produce L-
20 threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to α -amino- β -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α -methylserine, resistance to diaminosuccinic acid,
25 resistance to α -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a
30 partial and compensatable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

- resistance to L-aspartate, resistance to L-leucine,
resistance to L-phenylalanine, resistance to L-serine,
resistance to L-cysteine, resistance to L-valine,
sensitivity to fluoropyruvate, defective threonine
5 dehydrogenase, optionally an ability for sucrose
utilization, enhancement of the threonine operon,
enhancement of homoserine dehydrogenase I-aspartate kinase
I, preferably of the feed back resistant form, enhancement
of homoserine kinase, enhancement of threonine synthase,
10 enhancement of aspartate kinase, optionally of the feed
back resistant form, enhancement of aspartate semialdehyde
dehydrogenase, enhancement of phosphoenol pyruvate
carboxylase, optionally of the feed back resistant form,
enhancement of phosphoenol pyruvate synthase, enhancement
15 of transhydrogenase, enhancement of the RhtB gene product,
enhancement of the RhtC gene product, enhancement of the
YfiK gene product, enhancement of a pyruvate carboxylase,
and attenuation of acetic acid formation.

- It has been found that microorganisms of the
20 Enterobacteriaceae family produce L-amino acids, in
particular L-threonine, in an improved manner after
enhancement, in particular over-expression, of at least one
or more of the genes chosen from the group consisting of
ahpC and ahpF.

- 25 The use of endogenous genes is in general preferred.
"Endogenous genes" or "endogenous nucleotide sequences" are
understood as meaning the genes or nucleotide sequences
present in the population of a species.

- The nucleotide sequences of the genes of *Escherichia coli*
30 belong to the prior art and can also be found in the genome
sequence of *Escherichia coli* published by Blattner et al.
(*Science* 277: 1453-1462 (1997)).

The following information on the ahpC gene and ahpF gene is
known, inter alia, from the prior art:

ahpC gene:

Description: C22 subunit of alkyl hydroperoxide
reductase; detoxification of hydroperoxides

EC No.: 1.6.4.-

- 5 Reference: Ferrante et al.; Proceedings of the
National Academy of Sciences USA 92(17):
7617-7621 (1995) Poole and Ellis;
Biochemistry 35(1): 56-64 (1996) Nishiyama
et al.; Journal of Bacteriology 183(8):
10 2431-2438 (2001)

Accession No.: AE000166

ahpF gene:

Description: F52a subunit of alkyl hydroperoxide
reductase; detoxification of hydroperoxides

- 15 Reference: Ferrante et al.; Proceedings of the
National Academy of Sciences USA 92(17):
7617-7621 (1995) Poole and Ellis;
Biochemistry 35(1): 56-64 (1996) Poole et
al.; European Journal of Biochemistry
20 267(20): 6126-6133 (2000)

Accession No.: AE000166

- The nucleic acid sequences can be found in the databanks of
the National Center for Biotechnology Information (NCBI) of
the National Library of Medicine (Bethesda, MD, USA), the
25 nucleotide sequence databank of the European Molecular
Biologies Laboratories (EMBL, Heidelberg, Germany or
Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima,
Japan).

- Alleles of at least one or more of the genes chosen from
30 the group consisting of ahpC and ahpF which result from the
degeneracy of the genetic code or due to "sense mutations"
of neutral function can furthermore be used.

To achieve an enhancement, for example, expression of the genes or the catalytic properties of the proteins can be increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the
5 corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is
10 additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme
15 protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of
20 the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190
25 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169
30 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in
35 Enterobacteriaceae, such as e.g. cloning vectors derived

from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)),
pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101
derivatives (Vocke and Bastia, Proceedings of the National
Academy of Sciences USA 80(21): 6557-6561 (1983)) can be
5 used. A strain transformed with a plasmid vector, wherein
the plasmid vector carries at least one or more of the
genes chosen from the group consisting of *ahpC* and *ahpF* or
nucleotide sequences which code for them, can be employed
in a process according to the invention.

10 It is also possible to transfer mutations which affect the
expression of the particular gene into various strains by
sequence exchange (Hamilton et al. (Journal of Bacteriology
171: 4617 - 4622 (1989))), conjugation or transduction.

It may furthermore be advantageous for the production of L-
15 amino acids, in particular L-threonine, with strains of the
Enterobacteriaceae family to enhance one or more enzymes of
the known threonine biosynthesis pathway or enzymes of
anaplerotic metabolism or enzymes for the production of
reduced nicotinamide adenine dinucleotide phosphate, in
20 addition to the enhancement of one or more of the genes
chosen from the group consisting of *ahpC* and *ahpF*.

Thus, for example, one or more of the genes chosen from the
group consisting of

- the *thrABC* operon which codes for aspartate kinase,
25 homoserine dehydrogenase, homoserine kinase and
threonine synthase (US-A-4,278,765),
- the *pyc* gene which codes for pyruvate carboxylase (DE-A-
19 831 609),
- the *pps* gene which codes for phosphoenol pyruvate
30 synthase (Molecular and General Genetics 231(2): 332-336
(1992)),

- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- 5 • the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1
10 013 765),
- the thrE gene of Corynebacterium glutamicum which codes for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23:
15 199-209 (1983)),
- the dps gene which codes for the global regulator Dps (Genes & Development 6(12B): 2646-2654 (1992), Accession No. AE000183),
- the hns gene which codes for the DNA-binding protein
20 HLP-II (Molecular and General Genetics 212(2): 199-202 (1988), Accession No. AE000222),
- the lrp gene which codes for the regulator of the leucine Lrp regulon and high-affinity transport systems of branched-chain amino acids (Journal of Biological
25 Chemistry 266(17): 10768-10774 (1991), Accession No. AE000191),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994), Accession No. AE000172),

- the fba gene which codes for fructose bisphosphate aldolase (Biochemical Journal 257: 529-534 (1989), Accession No. AE000376),
- 5 • the ptsG gene which codes for the glucose-specific IIBC component of the phosphotransferase system PTS (Journal of Biological Chemistry 261(35): 16398-16403 (1986), Accession No. AE000210),
- 10 • the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 15 • the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 20 • the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- the mopB gene which codes for chaperone GroES (Journal of Biological Chemistry 261(26): 12414-12419 (1986), Accession No. AE000487),

can be enhanced, in particular over-expressed.

25 The use of endogenous genes is in general preferred.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of one or more of the genes chosen from the group consisting of ahpC and ahpF, for one or more of the
30 genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- 5 • the gene product of the open reading frame (orf) yjfa (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) ytfp (Accession Number AAC77179 of the National Center for
- 10 Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- the poxB gene which codes for pyruvate oxidase (Nucleic
- 15 Acids Research 14(13): 5449-5460 (1986)),
- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and
- 20 Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene, and
- 25 • the rpoS gene which codes for the sigma³⁸ factor (WO 01/05939) and is also known under the name of the katF gene,

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak
5 promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of
10 the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

15 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of one or more of the genes chosen from the group consisting of *ahpC* and *ahpF*, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing
20 Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed
25 batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction
30 to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General
5 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower
10 oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as
15 a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium
20 phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-
25 containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be
30 employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

5 Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or

10 oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually

15 reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by

20 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine,

25 L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in

30 Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory

Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of *Escherichia coli* is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America (1989) 86: 2172-2175).

The incubation temperature for the preparation of strains and transformants is 37°C.

Example 1

Construction of the expression plasmid pTrc99AahpCF

- 10 The *ahpC* and *ahpF* genes from *E. coli* K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequences of the *ahpC* and *ahpF* genes in *E. coli* K12 MG1655 (Accession Number AE000166, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for *Xba*I is chosen for the *ahpCF*1 primer and the recognition sequence for *Hind*III for the *ahpCF*2 primer, which are marked by underlining in the nucleotide sequence shown below:

*ahpCF*1: 5' - GCATCTAGACGATAACACGGAGGAAG - 3' (SEQ ID No. 1)

*ahpCF*2: 5' - GCTAAGCTTTTGCAGGTGAATC - 3' (SEQ ID No. 2)

- The chromosomal *E. coli* K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 2400 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is cleaved with the restriction enzymes *Xba*I and *Hind*III and

ligated with the vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which has been digested with the enzymes XbaI and HindIII. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the
5 ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRI and MluI. The plasmid is called pTrc99AahpCF (figure 1).

10 Example 2

Preparation of L-threonine with the strain
MG442/pTrc99AahpCF

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited
15 as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AahpCF described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar
20 with 50 µg/ml ampicillin. The strains MG442/pTrc99AahpCF and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 g/l KH_2PO_4 , 1 g/l NH_4Cl , 0.1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/l
25 glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(\text{NH}_4)_2\text{SO}_4$, 1 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g/l
30 CaCO_3 , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l $(\text{NH}_4)_2\text{SO}_4$, 2 g/l KH_2PO_4 , 1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 30 g/l CaCO_3 , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

| Strain | OD (660 nm) | L-Threonine g/l |
|--------------------|----------------|--------------------|
| MG442 | 5.6 | 1.4 |
| MG442/pTrc99A | 3.8 | 1.3 |
| MG442/pTrc99AahpCF | 5.4 | 2.6 |

20 Brief Description of the Figure:

- Figure 1: Map of the plasmid pTrc99AahpCF containing the ahpC and ahpF genes.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- Amp: Ampicillin resistance gene
- 5 • lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- ahpC: Coding region of the ahpC gene
- ahpF: Coding region of the ahpF gene
- 10 • 5S: 5S rRNA region
- rrnBT: rRNA terminator region

The abbreviations for the restriction enzymes have the following meaning

- EcoRI: Restriction endonuclease from Escherichia coli
15 RY13
- HindIII: Restriction endonuclease from Haemophilus influenzae
- MluI: Restriction endonuclease from Micrococcus luteus
IFO 12992
- 20 • XbaI: Restriction endonuclease from Xanthomonas campestris

What is claimed is:

1. Process for the preparation of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
 - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of *ahpC* and *ahpF* or the nucleotide sequences which code for these is
10 (are) enhanced, in particular over-expressed,
 - b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
 - c) isolation of the desired L-amino acid, constituents
15 of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof optionally remaining in the product.
2. Process according to claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are
20 employed.
3. Process according to claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 25 4. Process according to claim 1, wherein the expression of the polynucleotide(s) which code(s) for one or more of the genes chosen from the group consisting of *ahpC* and *ahpF* is increased.
5. Process according to claim 1, wherein the regulatory
30 and/or catalytic properties of the polypeptides

(proteins) for which the polynucleotides *ahpC* and *ahpF* code are improved or increased.

6. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 6.1 the *thrABC* operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
 - 6.2 the *pyc* gene which codes for pyruvate carboxylase,
 - 6.3 the *pps* gene which codes for phosphoenol pyruvate synthase,
 - 6.4 the *ppc* gene which codes for phosphoenol pyruvate carboxylase,
 - 6.5 the *pntA* and *pntB* genes which code for transhydrogenase,
 - 6.6 the *rhtB* gene which imparts homoserine resistance,
 - 6.7 the *mgo* gene which codes for malate:quinone oxidoreductase,
 - 6.8 the *rhtC* gene which imparts threonine resistance,
 - 6.9 the *thrE* gene which codes for the threonine export protein,
 - 6.10 the *gdhA* gene which codes for glutamate dehydrogenase,

- 6.11 the hns gene which codes for the DNA-binding protein HLP-II,
- 6.12 the lrp gene which codes for the regulator of the leucine Lrp regulon,
- 5 6.13 the pgm gene which codes for phosphoglucomutase,
- 6.14 the fba gene which codes for fructose bisphosphate aldolase,
- 10 6.15 the ptsG gene which codes for the glucose-specific IIBC component,
- 6.16 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 15 6.17 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 6.18 the crr gene which codes for the glucose-specific IIA component,
- 6.19 the mopB gene which codes for chaperone GroES,
- 20 6.20 the dps gene which codes for the global regulator Dps,

is or are enhanced, in particular over-expressed, are fermented.

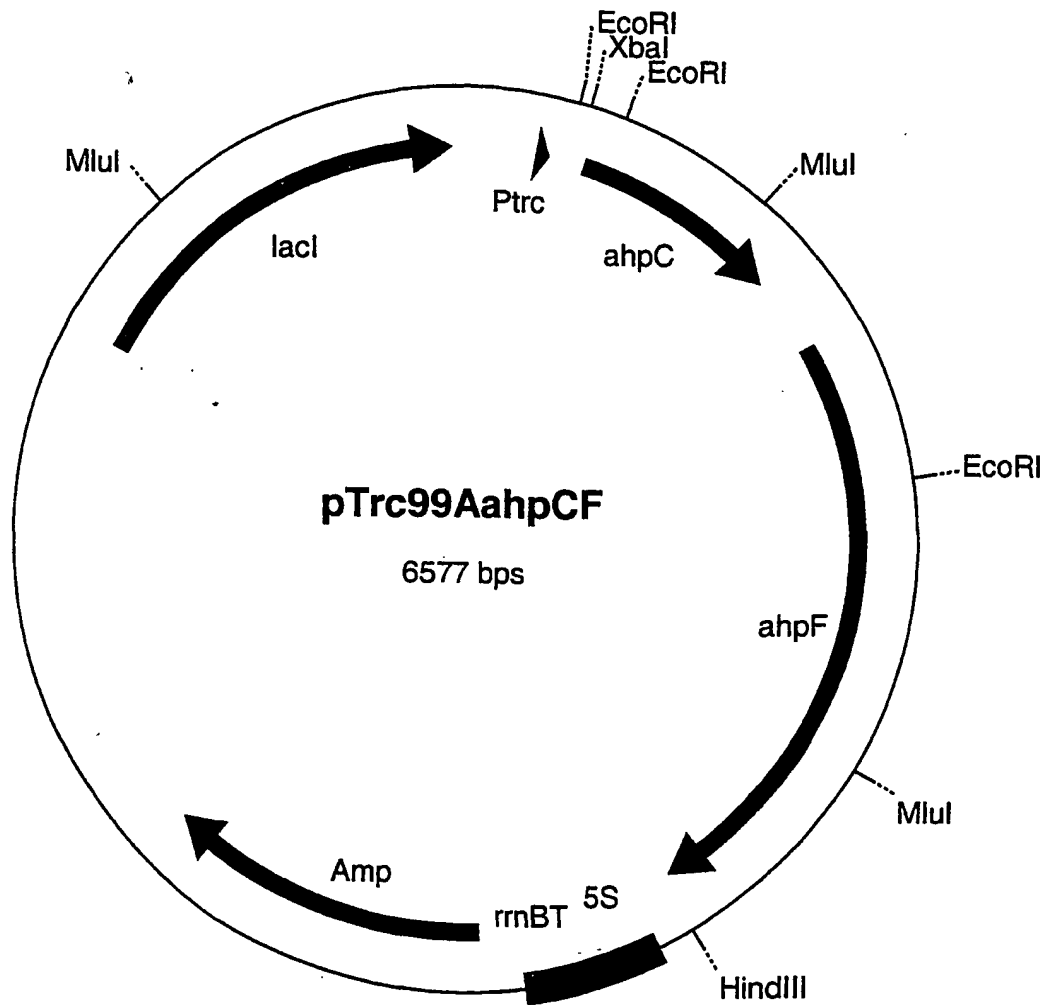
7. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 25

- 7.1 the tdh gene which codes for threonine dehydrogenase,

- 7.2 the mdh gene which codes for malate dehydrogenase,
- 7.3 the gene product of the open reading frame (orf) yjfA,
- 5 7.4 the gene product of the open reading frame (orf) ytfP,
- 7.5 the pckA gene which codes for phosphoenol pyruvate carboxykinase,
- 7.6 the poxB gene which codes for pyruvate oxidase,
- 10 7.7 the aceA gene which codes for isocitrate lyase,
- 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
- 7.9 the fruR gene which codes for the fructose repressor,
- 15 7.10 the rpoS gene which codes for the sigma³⁸ factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Figure 1:



SEQUENCE PROTOCOL

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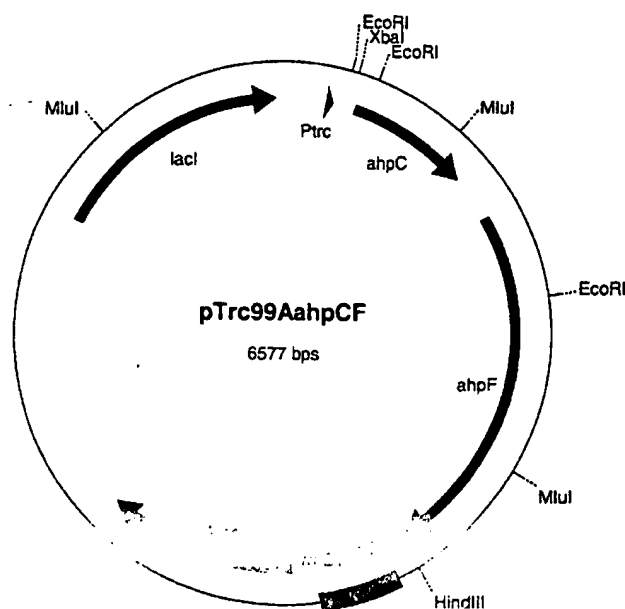
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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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International Application No

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | US 4 278 765 A (DEBBOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14) cited in the application the whole document --- | 1-7 |
| A | US 5 807 735 A (COYLE CATHERINE L ET AL) 15 September 1998 (1998-09-15) the whole document --- | 1-7 |
| A | EP 0 994 190 A (AJINOMOTO KK) 19 April 2000 (2000-04-19) the whole document --- | 1-7 |
| | -/-- | |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| A | SAWERS G: "TH ANAEROBIC DEGRADATION OF L-SERINE AND L-THREONINE IN ENTEROBACTERIA: NETWORKS AND PATHWAYS AND REGULATORY SIGNALS" ARCHIVES OF MICROBIOLOGY, BERLIN, DE, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document | 1-7 |
| E | WO 03 004598 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 & WO 03 004664 A 16 January 2003 (2003-01-16) & WO 03 004665 A & WO 03 004669 A & WO 03 004670 A & WO 03 004671 A & WO 03 004674 A & WO 03 004675 A | 6 |
| T | WO 03 004662 A (DEGUSSA) 16 January 2003 (2003-01-16) the whole document | 1-7 |

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/06560

| Patent document cited in search report | | Publication date | Patent family member(s) | Publication date |
|---|---|---------------------|---|--|
| US 4278765 | A | 14-07-1981 | SU 875663 A1 HU 190999 B | 15-09-1982 28-12-1986 |
| US 5807735 | A | 15-09-1998 | NONE | |
| EP 0994190 | A | 19-04-2000 | RU 2144564 C1 AU 4755099 A BR 9904955 A CN 1254014 A EP 0994190 A2 JP 2000116390 A KR 2000029006 A SK 140899 A3 US 6303348 B1 US 2002102670 A1 US 2002058314 A1 ZA 9906042 A | 20-01-2000 20-04-2000 12-12-2000 24-05-2000 19-04-2000 25-04-2000 25-05-2000 16-05-2000 16-10-2001 01-08-2002 16-05-2002 04-04-2000 |
| WO 03004598 | A | 16-01-2003 | DE 10132946 A1 WO 03004663 A2 WO 03004669 A2 WO 03004674 A2 WO 03004670 A2 WO 03004664 A2 WO 03004598 A2 WO 03004665 A2 WO 03004671 A2 WO 03004675 A2 | 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 |
| WO 03004662 | A | 16-01-2003 | DE 10132945 A1 WO 03004662 A2 US 2003017556 A1 | 16-01-2003 16-01-2003 23-01-2003 |

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